

## A Functional NADPH Oxidase Prevents Caspase Involvement in the Clearance of Phagocytic Neutrophils<sup>†</sup>

Rachel P. Wilkie,<sup>1</sup> Margret C. M. Vissers,<sup>1</sup> Mike Dragunow,<sup>2</sup> and Mark B. Hampton<sup>1\*</sup>

Free Radical Research Group, Department of Pathology, Christchurch School of Medicine and Health Sciences, University of Otago, Christchurch, New Zealand,<sup>1</sup> and Department of Pharmacology and Clinical Pharmacology and National Research Centre for Growth and Development, University of Auckland, Auckland, New Zealand<sup>2</sup>

Received 18 December 2006/Returned for modification 14 February 2007/Accepted 3 April 2007

**Neutrophils play a prominent role in host defense. Phagocytosis of bacteria leads to the formation of an active NADPH oxidase complex that generates reactive oxygen species for bactericidal purposes. A critical step in the resolution of inflammation is the uptake of neutrophils by macrophages; however, there are conflicting reports on the mechanisms leading to the apoptosis of phagocytic neutrophils. The aim of this study was to clarify the role of effector caspases in these processes. Caspase activity was measured by DEVDase activity assays or immunofluorescence detection of active caspase-3. With normal human and wild-type murine neutrophils there was no caspase activation following phagocytosis of *Staphylococcus aureus*. However, caspase activity was observed in phagocytic neutrophils with a defective NADPH oxidase, including neutrophils isolated from X-linked gp91<sup>phox</sup> knockout chronic granulomatous disease mice. These results indicate that a functional NADPH oxidase and the generation of oxidants in the neutrophil phagosome prevent the activation of the cytoplasmic caspase cascade.**

Neutrophils play an integral role in the eradication of pathogens from the body. These cells contain a range of toxic compounds, and it is essential that they remain intact without releasing intracellular contents that might damage host tissue. Following maturation and release from the bone marrow, the circulating neutrophil has a life span of 1 to 2 days before it undergoes apoptosis (26). This process has been well characterized and includes caspase activation, phosphatidylserine (PS) exposure, and phagocytosis of the dying cell (8, 9, 27). The rate of this spontaneous apoptosis is susceptible to modulation. If neutrophils are exposed to proinflammatory cytokines, such as interleukins, granulocyte-macrophage colony-stimulating factor, or bacterial lipopolysaccharide, the apoptotic process is delayed (3, 5), thereby prolonging the neutrophil life span and presumably the ability of neutrophils to contribute to pathogen removal.

At an inflammatory site, neutrophils ingest pathogens into intracellular compartments called phagosomes, where killing occurs (11). Phagocytic neutrophils are themselves ingested by macrophages before they disintegrate. However, the mechanism of apoptosis in phagocytic neutrophils appears to be more complicated and controversial than the spontaneous apoptosis of unstimulated cells. Both acceleration (18, 21, 23, 34–36) and retardation (1, 23, 31) of neutrophil apoptosis in actively phagocytosing neutrophils have been reported.

Stimulated neutrophils generate vast amounts of oxidants upon activation of the NADPH oxidase membrane complex within the phagosome (11), and several studies have demonstrated that these oxidants promote the onset of neutrophil

apoptosis (7, 14, 23, 24, 28, 34). However, the role of the caspases in this process is unclear. These enzymes play a key role in the cascade of proteolytic cleavage that occurs during apoptosis. Their activation is redox sensitive (12, 13), and we have previously shown that triggering the oxidative burst with phorbol myristate acetate (PMA) blocks caspase activation (8). The situation during phagocytosis, however, differs from that with the artificial PMA stimulus. In particular, oxidant production is generally considered to be restricted to the internal phagosome during phagocytosis, compared with NADPH oxidase activation over the entire plasma membrane with PMA (32). The duration and extent of the oxidative burst also vary in the two systems. There have been two reports suggesting that in contrast to the PMA model, caspases are activated during the apoptosis of phagocytic neutrophils (24, 35).

In this study we investigated the effect of NADPH oxidase on caspase activation in phagocytic neutrophils. The model used involved phagocytosis of *Staphylococcus aureus* by neutrophils isolated from X-linked gp91<sup>phox</sup> knockout mice with a nonfunctional NADPH oxidase. We found that while neutrophil oxidant generation is required for PS exposure and uptake by macrophages, the oxidants clearly prevented caspase activation. Indeed, inhibition of the oxidative burst led to enhanced caspase activation but decreased clearance by macrophages. This indicates that the clearance of actively phagocytosing neutrophils is a caspase-independent process.

### MATERIALS AND METHODS

**Materials.** *S. aureus* strain 502a (ATCC 27217) was obtained from the New Zealand Communicable Disease Centre (Porirua, New Zealand), and Trypticase soy broth was obtained from Becton Dickinson (Cockeysville, MD). Cell culture medium was supplied by Gibco-BRL (Grand Island, NY), and normal mouse serum was supplied by Biomed (Foster City, CA). The caspase-3 substrate DEVD-AMC was obtained from Peptide Institute Inc. (Osaka, Japan), and the caspase inhibitor z-VAD-fmk was obtained from Enzyme Systems Products (Livermore, CA). An annexin V-fluorescein isothiocya-

\* Corresponding author. Mailing address: Department of Pathology, Christchurch School of Medicine and Health Sciences, P.O. Box 4345, Christchurch, New Zealand. Phone: 64 3 3641524. Fax: 64 3 3641083. E-mail: mark.hampton@chmeds.ac.nz.

<sup>†</sup> Published ahead of print on 16 April 2007.

nate (FITC) Apoptest kit was obtained from Dakocytomation (Glostrup, Denmark [for NeXins Research, Kattendijke, The Netherlands]). Monoclonal rabbit anti-human cleaved caspase-3 antibody was obtained from Cell Signaling Technology Inc. (Beverly, MA), and Diff-Quik was obtained from Dade Behring AG (Dudingen, Switzerland). Diphenyleneiodonium (DPI), paraformaldehyde, *o*-dianisidine, and thioglycolate were all obtained from Sigma Chemical Co. (St. Louis, MO).

**Preparation of *S. aureus*.** *S. aureus* was cultured overnight in Trypticase soy broth, harvested by centrifugation, washed, and resuspended in Hanks balanced salt solution (HBSS) (10 mM phosphate buffer [pH 7.4] containing 140 mM NaCl, 0.5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 1 mg/ml glucose). Bacterial cell density was measured spectrophotometrically at 550 nm, and the cell number was calculated using a standard curve based on CFU counts. Heat-killed propidium iodide (PI)-labeled *S. aureus* was prepared by heating 10<sup>9</sup> *S. aureus* cells/ml at 90°C for 30 min before adding 2 µg/ml PI. Bacteria were then pelleted, the supernatant was discarded to remove excess PI, and the cells were resuspended in HBSS. All bacteria were opsonized with 10% autologous human serum or commercially obtained mouse serum and rotated end over end for 20 min at 37°C immediately before addition to neutrophils at defined ratios.

**Isolation of human and mouse neutrophils.** Neutrophils were isolated from heparinized peripheral blood of healthy adult donors under sterile conditions by Ficoll-Hypaque centrifugation, dextran sedimentation, and hypotonic lysis. Mouse neutrophils were obtained from the peritoneal cavities of X-linked gp91<sup>phox</sup> knockout and C57BL/6 wild-type mice 18 h after injection of 1 ml of 4% thioglycolate, as approved by the University of Otago Animal Ethics Committee. Cells collected in HBSS supplemented with 0.1% bovine serum albumin were spun through 1.5 ml of Ficoll-Hypaque at 1,000 × *g* for 20 min to concentrate the neutrophils.

**Neutrophil stimulation.** Human or murine neutrophils (10<sup>7</sup> cells/ml) were incubated at 37°C in RPMI 1640 medium with 10% autologous or mouse serum for 10 min in the presence or absence of 10 µM DPI. DPI-pretreated or untreated neutrophils were then incubated at 37°C in 5% CO<sub>2</sub> for up to 5 h with occasional mixing with opsonized *S. aureus* to obtain a ratio of 2, 10, 20, or 50 bacteria per neutrophil. At various intervals cells were harvested, and cytopsins were prepared by first removing any nonphagocytosed bacteria by differential centrifugation at 100 × *g* (15), followed by cytocentrifugation for 5 min at 250 rpm onto high-binding microscope glass slides. Each spot was fixed with 4% (wt/vol) paraformaldehyde (pH 7.4) and stained with Diff-Quik. The number of cells that had phagocytosed *S. aureus* was determined, and the morphology was observed.

**Fluorometric analysis of effector caspase activity.** Neutrophils (10<sup>6</sup> cells) that were untreated or pretreated with DPI were incubated without *S. aureus* or with *S. aureus* at ratios of 1:2, 1:10, 1:20, and 1:50 and centrifuged at selected times to remove the medium. The pellets were washed with phosphate-buffered saline, and caspase-3-like activity was determined by assessment of DEVD-AMC cleavage. Briefly, pellets were transferred to a microtiter plate and resuspended in 100 µl (final volume) of a caspase buffer solution containing 100 mM HEPES, 10% sucrose, 0.1% NP-40, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and 5 mM dithiothreitol (pH 7.2) supplemented with 50 µM of the fluorogenic peptide substrate Ac-DEVD-AMC. The cleavage of the caspase substrate was monitored over a 30-min period at 37°C in a Fluoroscan II plate reader using an excitation wavelength of 390 nm and an emission wavelength of 460 nm.

**Immunofluorescence analysis and quantification of caspase-3.** Neutrophils (10<sup>7</sup> cells/ml) pretreated with or without DPI were incubated with or without PI-labeled *S. aureus* (1:20) for 3 h at 37°C. Nonphagocytic neutrophils and phagocytic neutrophils at a cell/bacterium ratio of 1:20 were cytospun onto the same microscope slide to obtain a mixed population of cells. The active form of caspase-3 was detected by immunofluorescence using a previously described method (4), a monoclonal rabbit anti-human cleaved caspase-3 antibody, and FITC-conjugated goat anti-rabbit immunoglobulin G. A Leitz Aristoplan fluorescence microscope was used to capture images of cleaved caspase-3 immunofluorescence (green fluorescence) and red fluorescent PI-labeled neutrophils. Color composite images were then processed using a high-throughput image analysis program (Metamorph V.6.2.6; Molecular Devices) to determine the average fluorescence intensity of cleaved caspase-3 staining in phagocytic (red fluorescence) and nonphagocytic (no red fluorescence) neutrophils. A "journal" was written to automatically color separate the red and green channels, convert each image into a 16-bit image format for analysis, and then insert each pair of 16-bit images into the Cell Scoring application of Metamorph. The Cell Scoring algorithm determined the average fluorescence intensity of cleaved caspase-3 staining in phagocytic (red fluorescence) and nonphagocytic (no red fluorescence) neutrophils and automatically logged the data into Excel spreadsheets.

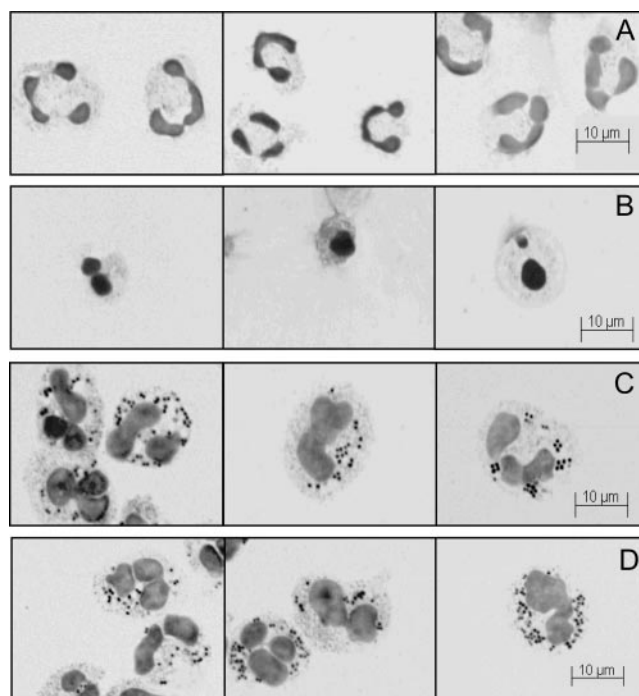


FIG. 1. Phagocytosis of *S. aureus* by human neutrophils and subsequent changes in morphology. Photographs from a representative experiment for cytopsins of human unstimulated neutrophils at 3 h (A), of untreated neutrophils after 48 h of incubation (B), of neutrophils with *S. aureus* at 3 h (C), and of neutrophils with *S. aureus* at 5 h (D).

The advantages of this method for quantifying imaging data are that it is fully automated, objective, and standardized. Further information about this high-throughput image analysis platform can be found at <http://www.health.auckland.ac.nz/pharmacology/discovery-1/>.

**Exposure of PS.** The externalization of PS was assessed using annexin V-FITC according to the protocol outlined in the Apoptest-FITC kit. Untreated or DPI-pretreated wild-type or chronic granulomatous disease (CGD) murine neutrophils were incubated with *S. aureus* at a 1:20 ratio in the presence or absence of the caspase inhibitor z-VAD-fmk (10 µM) for 4 h at 37°C. Binding was assessed using calcium-free HEPES buffer with EGTA (10 mM HEPES-NaOH [pH 7.4], 150 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA) to confirm that fluorescence increases were due to annexin V binding, which is calcium dependent, and was also assessed without any addition of annexin V-FITC. Flow cytometry was performed with a FACS Vantage from Becton Dickinson (San Jose, CA), and the data were analyzed using CellQuest software. Ten thousand events were analyzed, and both the geometric mean fluorescence of viable cells and the percentage of cells displaying more than 10 fluorescent units were recorded. PI-positive necrotic neutrophils (10 to 17%) were gated out of the final analysis.

**Uptake of phagocytic neutrophils by macrophages.** Human monocyte-derived macrophages were prepared by adhering the peripheral blood monocyte layer after Ficoll-Hypaque centrifugation for 2 h on a 24-well tissue culture plate (5 × 10<sup>6</sup> cells/well) (14). Following vigorous washing with HBSS, adherent monocytes were cultured in Iscove's modified Dulbecco's medium with 10% serum for 7 days. Mouse neutrophils (10<sup>6</sup> cells) that were not treated or were pretreated with DPI were incubated with or without *S. aureus* (1:20) for 4 h at 37°C, harvested, and resuspended in 500 µl HBSS. Iscove's modified Dulbecco's medium was removed from the 7-day macrophage wells and replaced with the neutrophils in HBSS, and the preparations were incubated for 1 h at 37°C. Wells were then fixed with 4% paraformaldehyde for 10 min, and neutrophils were visualized with a myeloperoxidase stain (1 mM *o*-dianisidine, 5 mM H<sub>2</sub>O<sub>2</sub>, 50 mM sodium phosphate buffer; pH 6). The number of neutrophils phagocytosed per 100 macrophages was determined.

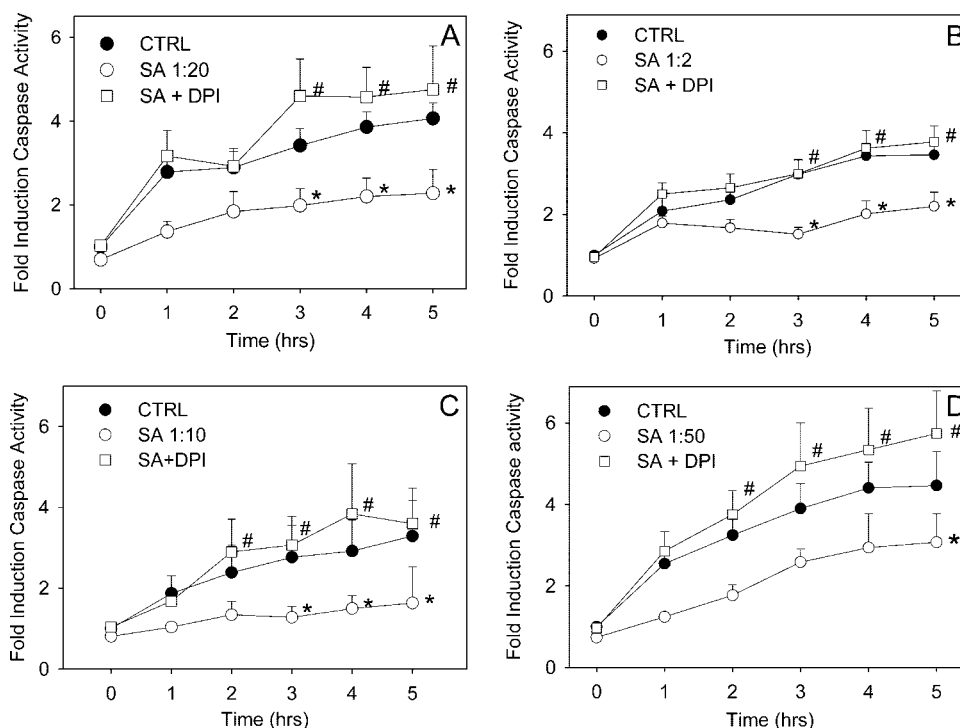


FIG. 2. Caspase-3 activation is blocked by NADPH oxidase activity in phagocytic human neutrophils. Caspase activity was assessed hourly in neutrophils incubated alone (CTRL), in neutrophils incubated with *S. aureus* (SA) at ratios of 1:20 (A), 1:2 (B), 1:10 (C), and 1:50 (D), and in neutrophils treated with DPI prior to coinocubation with *S. aureus* (SA + DPI). The active caspase-3 activity was measured by monitoring the arbitrary units of fluorescence liberated following cleavage of the fluorogenic peptide substrate DEVD-AMC. The means and standard errors of the means of four to nine experiments are shown. An asterisk indicates that the *P* value is <0.05 for a comparison with the control, and a number sign indicates that the *P* value is <0.05 for a comparison with phagocytic neutrophils with a functional NADPH oxidase.

**Statistics.** Statistical analysis was performed with the SigmaStat software package from Jandel Scientific (SPSS Science, Chicago, IL) using repeated-measures analysis of variance, followed by the Bonferroni test for multiple comparisons.

## RESULTS

**Caspase activation in human phagocytic neutrophils.** We have previously established that artificial stimulation of the oxidative burst in neutrophils by a phorbol ester can prevent activation of the redox-sensitive caspases (8). To determine whether this phenomenon is physiological, we investigated the effect of NADPH oxidase-derived oxidants on caspase activity in neutrophils phagocytosing bacteria. In our system neutrophils were incubated with opsonized *S. aureus* at ratios ranging from 2 to 50 bacteria per neutrophil and harvested at various intervals. We have previously demonstrated that with continual mixing *S. aureus* is rapidly internalized (half-life, 9 min [15]). In this system we used occasional mixing to prevent excessive neutrophil damage, and all neutrophils had bacteria in their cytoplasm within 30 min. None of the inhibitors used in this study had any effect on the kinetics of uptake. Phagocytic neutrophils lost their characteristic multilobed nuclei at 3 h (Fig. 1C) and instead displayed nuclei that were swollen and irregular in shape. These nuclei were very different from the shrunken and condensed nuclei in neutrophils that had undergone spontaneous apoptosis at 48 h (Fig. 1B). At 5 h swollen nuclei were still apparent (Fig. 1D), and the results at later

time points were impossible to assess because the phagocytic neutrophils were too fragile to survive the cytospin process.

There was a three- to fourfold increase in the activity of effector caspases measured with the fluorescent substrate DEVD-AMC in unstimulated human neutrophils over 5 h (Fig. 2A). This reflected initiation of spontaneous apoptosis. In contrast, caspase activation was delayed following phagocytosis of *S. aureus* (Fig. 2A). DPI treatment of phagocytic neutrophils resulted in enhanced caspase activation (Fig. 2A). DPI alone had no effect on the rate of spontaneous caspase activity. *S. aureus* itself had no detectable caspase activity, and it was also not able to inhibit caspases, as demonstrated by the significant levels of caspase activity seen in DPI-treated phagocytic neutrophils. Addition of the supernatant of cultured *S. aureus* had no influence on the levels of caspase activation observed in nonphagocytic neutrophils (not shown), demonstrating that the inhibition of caspase activity observed in neutrophils coinocubated with *S. aureus* was indeed due to phagocytosis and not due to a soluble factor secreted by the bacteria.

We explored the effects of different neutrophil/*S. aureus* ratios on caspase activation. After 1 h of incubation ratio-dependent inhibition of caspase activity was observed (Fig. 3). However, over the 5-h time course there was little difference between ratios (Fig. 2B to D). Cytospins at 3 h showed that although the cultures began at a ratio of 1:2, bacterial growth during this period meant that the ratio was higher at later time

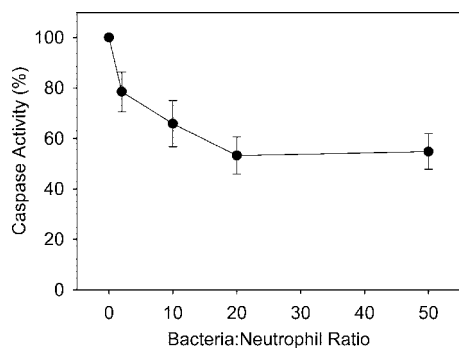


FIG. 3. Dose-dependent inhibition of caspase activity. Caspase activity in neutrophils incubated with *S. aureus* at ratios of 1:2, 1:10, 1:20, and 1:50 after 1 h of incubation is expressed as a percentage of the activity expressed at 1 h in unstimulated neutrophils.

points (not shown). Phagocytic neutrophils pretreated with DPI at all ratios showed levels of caspase activity either equivalent to or slightly greater than the levels of cells undergoing spontaneous apoptosis (Fig. 2A to D).

**Immunofluorescence analysis of active caspase-3.** To confirm the caspase activity assays, we used an immunofluorescence technique that detects the active form of caspase-3 in individual cells. Neutrophils were incubated with or without PI-labeled *S. aureus* for 3 h, and the two populations of cells were mixed immediately prior to cytospin to enable direct comparison between phagocytic and unstimulated neutrophils. Nonphagocytic neutrophils undergoing spontaneous apoptosis showed strong active caspase-3 staining within the cytoplasm (Fig. 4A). In contrast, neutrophils that had phagocytosed PI-labeled *S. aureus* displayed reduced cytosolic active caspase-3 staining (Fig. 4A). Inhibition of the NADPH oxidase by DPI, however, resulted in the phagocytic neutrophils demonstrating caspase-3 fluorescence staining equal to or greater than that of nonphagocytic cells (Fig. 4B).

To quantify these observations, we used a high-throughput image analysis program (Metamorph V.6.2.6; Molecular Devices) to determine the average fluorescence intensities of cleaved caspase-3 staining in PI-labeled phagocytic (red fluorescence) and nonphagocytic (no red fluorescence) neutrophils (Fig. 4C). The results of this objective analysis confirmed our initial observations and showed that there was a statistically significant reduction in cleaved caspase-3 in phagocytic neutrophils compared with nonphagocytic neutrophils, as well as a reversal of the suppression by DPI (Fig. 4C).

**Caspase activation in murine phagocytic neutrophils.** DPI is not a specific inhibitor of the NADPH oxidase and is known to have a variety of effects on cells (20, 25). To validate our finding that caspase-3 activation is blocked by NADPH oxidase-derived oxidants upon phagocytosis, we assessed caspase activation in phagocytic murine CGD neutrophils with a nonfunctional NADPH oxidase. Neutrophils from the mouse peritoneal cavity were incubated with opsonized *S. aureus* at a ratio of 1:20 and harvested at selected times. The extents of phagocytosis of *S. aureus* were comparable for wild-type and CGD murine neutrophils, and no apparent difference in nuclear morphology was observed (not shown).

There was a threefold increase in caspase-3 activation in

unstimulated wild-type murine neutrophils, which was blocked in the phagocytic neutrophils (Fig. 5A). When the oxidative burst was inhibited by DPI, increased caspase-3 activity was induced in phagocytic cells (Fig. 5A). Unstimulated CGD mouse neutrophils showed no spontaneous caspase-3 activation during the 5-h incubation, but following phagocytosis of *S. aureus* the levels of caspase activity were comparable to those of DPI-treated phagocytic wild-type neutrophils and increased more than fourfold (Fig. 5B). In combination, these results suggest that the process of phagocytosis can result in caspase activation but that an active NADPH oxidase prevents this from occurring.

**PS exposure and uptake of murine phagocytic neutrophils by macrophages.** We have previously shown by using pharmacological inhibition that a functional NADPH oxidase is important for PS exposure in human phagocytic neutrophils (14). Therefore, we sought to investigate PS externalization and neutrophil uptake by macrophages in our CGD mouse model, which we have now demonstrated to have elevated caspase activity. PS exposure was assessed by annexin V-FITC labeling in wild-type and CGD mouse neutrophils. Phagocytosis of *S. aureus* triggered a threefold increase in annexin V-FITC-positive wild-type murine neutrophils (Fig. 6B and D). This increase in fluorescence was not observed in the absence of annexin V-FITC or when a calcium-free buffer was used to prevent the binding of annexin V-FITC to PS (not shown). The increased PS exposure was blocked upon treatment with DPI (Fig. 6C and D). Similarly, neutrophils isolated from CGD mice did not express PS after phagocytosis of *S. aureus* (Fig. 6D), confirming the importance of a functional NADPH oxidase for PS externalization. The presence of the general caspase inhibitor z-VAD-fmk had no significant effect on the NADPH oxidase-dependent externalization of PS observed in phagocytic neutrophils (Fig. 6B).

The key apoptotic process leading to clearance of cells from an inflammatory site is uptake by macrophages. To determine whether macrophages were able to recognize and engulf the phagocytic cells, neutrophils from wild-type and CGD mice were coincubated with *S. aureus* and then layered onto human monocyte-derived macrophages. We found that macrophages actively engulfed wild-type neutrophils that had phagocytosed *S. aureus* and that this was also blocked by DPI treatment (Fig. 6E). Furthermore, we observed impaired uptake of phagocytic CGD mouse neutrophils by macrophages (Fig. 6E).

## DISCUSSION

Neutrophils undergo apoptosis following the phagocytosis and killing of pathogens, but it is not clear whether apoptosis proceeds via a conventional caspase-dependent pathway or an alternate pathway. Using enzymatic and immunofluorescence assays of effector caspases in phagocytic murine and human neutrophils, we demonstrated that caspase activation does not occur following phagocytosis. Caspases were detected only when the NADPH oxidase was inhibited or absent. However, it is known that the oxidative burst is critical for PS exposure and macrophage uptake. Therefore, caspase activation and the oxidative burst appear to be mutually exclusive events in the phagocytic neutrophil.

We have previously shown that sustained oxidant production



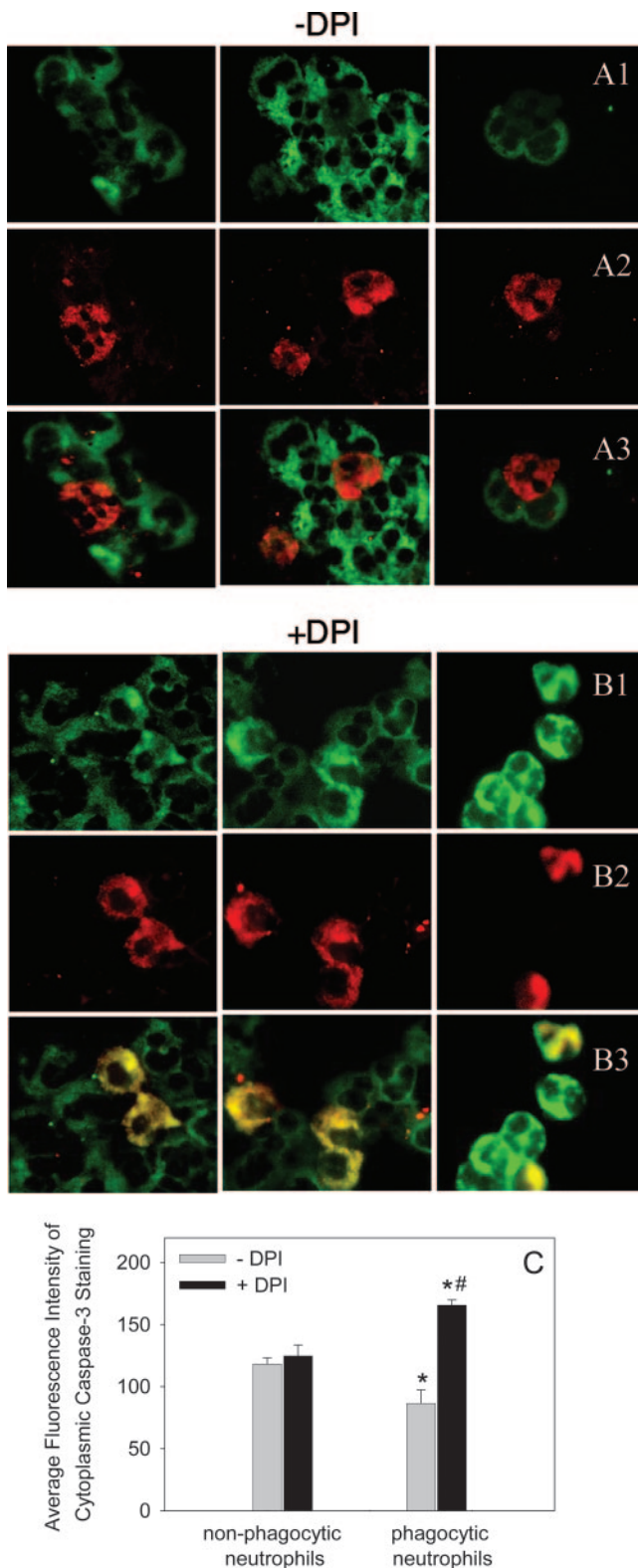


FIG. 4. Immunofluorescence staining for active caspase-3. Neutrophils were incubated either alone or with PI-labeled *S. aureus* (1:20) for 3 h, and then both phagocytic and nonphagocytic neutrophils were mixed and cytopun onto the same microscope slide. Cells were treated with a cleaved caspase-3 antibody, followed by a secondary antibody conjugated to FITC, and were visualized with a fluorescent microscope. Images of the green fluorescence (A1 and B1) showing positive

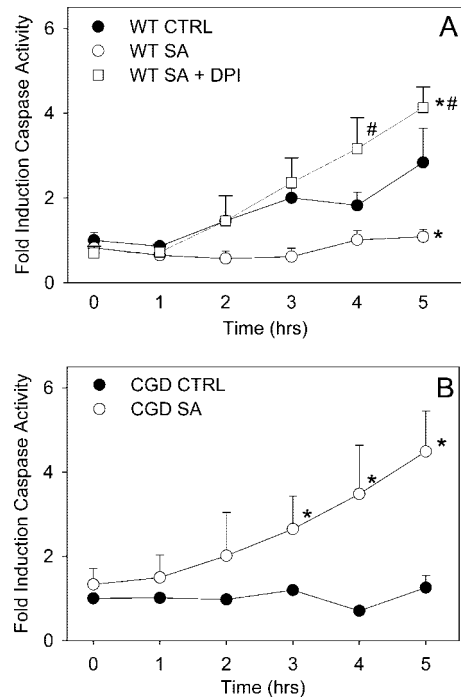


FIG. 5. Caspase-3 activation in phagocytic murine neutrophils. Neutrophils were incubated either alone (CTRL) or with *S. aureus* (SA) (1:20) or were treated with DPI prior to coincubation with *S. aureus* (SA + DPI). Caspase-3 activity was assessed hourly by monitoring the increase in fluorescence with excitation at 390 nm and emission at 460 nm following cleavage of the fluorogenic peptide substrate DEVD-AMC. The means and standard errors of the means of 3 to 10 experiments are shown. An asterisk indicates that the *P* value is  $<0.05$  for a comparison with the control, and a number sign indicates that the *P* value is  $<0.05$  for a comparison with phagocytic neutrophils with a functional NADPH oxidase. WT, wild type.

can interfere with caspase activation (12, 13), and the same phenomenon was observed in neutrophils stimulated with the artificial stimulus PMA (8). It is possible that phagosomal production and consumption of oxidants could spare cytoplasmic caspases, but our data clearly show that phagocytic cells have reduced caspase activity, as assessed by activity assays and

caspase-3 staining localized in the cytosol and the red fluorescence (A2 and B2) identifying phagocytic neutrophils were captured from the same field, and the pairs of images were superimposed (A3 and B3). Untreated phagocytic neutrophils (A2) surrounded by nonphagocytic neutrophils (A3) show negligible caspase-3 activity compared to the activity of the neighboring nonphagocytic cells (A1). When phagocytic neutrophils were pretreated with DPI (B1 to B3) to inhibit the NADPH oxidase, the phagocytic cells adjacent to nonphagocytic neutrophils (B3) displayed enhanced caspase activation (B1). The photographs are from representative experiments. (C) Quantification of caspase-3 fluorescence within untreated and phagocytic neutrophils with or without DPI treatment, analyzed using the Discovery-1 high-throughput and high-content screening machine. Fluorescence intensity was assessed in 300 cells from 27 photographs taken in three different experiments. An asterisk indicates that the *P* value is  $<0.05$  for a comparison with the control, and a number sign indicates that the *P* value is  $<0.05$  for a comparison with phagocytic neutrophils with a functional NADPH oxidase.

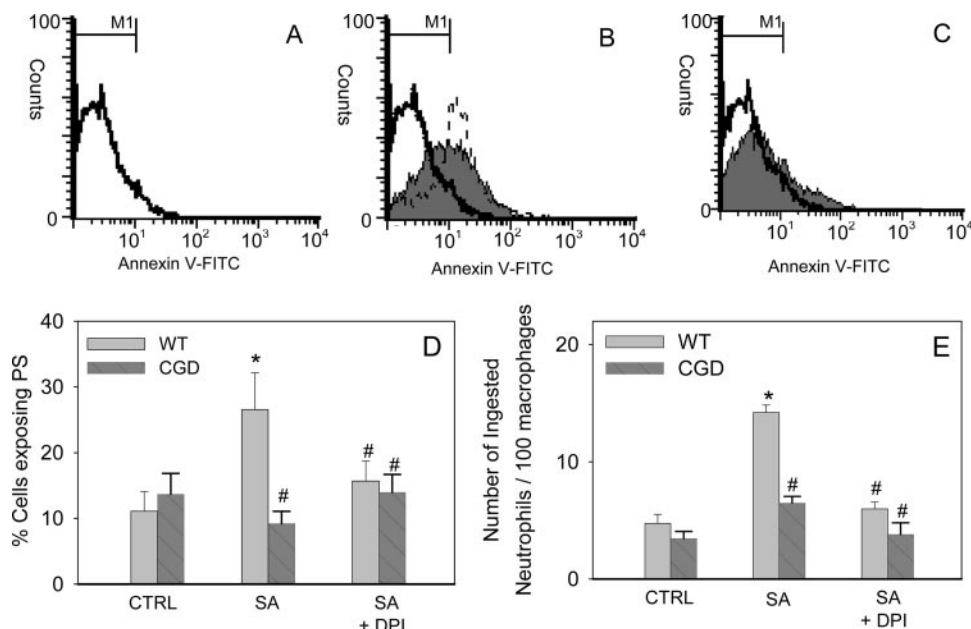


FIG. 6. Phagocytosis triggers PS exposure and uptake by macrophages in murine neutrophils and requires a functional NADPH oxidase. After 4 h of incubation with *S. aureus* (1:20) cells were stained with annexin V-FITC and PI (A to C). The flow cytometry histograms show the results of a single representative experiment with mouse wild-type neutrophils. (A) Unstimulated neutrophils; (B) neutrophils with *S. aureus* (shaded area); (C) DPI-treated neutrophils with *S. aureus* (shaded area). The results of treatment of neutrophils with *S. aureus* in the presence of the caspase inhibitor z-VAD-fmk are indicated by the dashed line in panel B. (D) Percentage of murine wild-type (WT) and CGD neutrophils exposing PS following incubation alone (CTRL), following incubation with *S. aureus* (SA), or following pretreatment with DPI (SA + DPI). (E) For macrophage uptake studies, murine neutrophils were incubated with *S. aureus*, harvested, and layered onto monocyte-derived macrophages, the medium was removed, and the wells were fixed and stained for myeloperoxidase with *o*-dianisidine, enabling visualization of neutrophils. The number of neutrophils phagocytosed per 100 macrophages was determined. The means and standard errors of the means of three to eight experiments are shown. An asterisk indicates that the *P* value is <0.05 for a comparison with the control, and a number sign indicates that the *P* value is <0.05 for a comparison with phagocytic neutrophils with a functional NADPH oxidase.

immunohistochemistry. Our results differ from the reports of Zhang et al. (35) and Perskvist et al. (24), which demonstrated that there was caspase activation in phagocytic neutrophils and suggested that this activity is a requirement for apoptosis (24, 35). The basis of the apparent discrepancy between the studies is unclear, although it might be related to the nature of the microorganism used to activate the neutrophils. We explored the effects of different neutrophil/bacterium ratios and saw no difference, and significant inhibition of caspase activation occurred even at low ratios. There was also no difference in the effect of phagocytosis on caspase activation due to live or dead bacteria, as demonstrated in our immunofluorescence study, where neutrophils phagocytosed heat-killed *S. aureus*. This rules out any contribution of killing defects in provoking the different responses. Others have reported that inhibition of the NADPH oxidase had no effect on the *Entamoeba*-induced cleavage of caspase-3 in human neutrophils (28) and that phagocytosis results in a reduction of caspase activity (31) and caspase-3 gene expression (2), thus supporting our results.

It is possible that phagocytic neutrophils secrete factors that dampen caspase activity in surrounding neutrophils. However, the immunofluorescence assay clearly illustrated that the NADPH oxidase-dependent block in caspase activation occurs in the individual phagocytic cells. DPI-treated or CGD neutrophils were the only phagocytic neutrophils to show significant caspase activity. However, there was no PS exposure or uptake of these cells. Furthermore, PS exposure in phagocytic

neutrophils occurred in the presence of a caspase inhibitor. This implies that caspases are not involved in the clearance of phagocytic neutrophils but that an oxidant-dependent event is crucial. This is consistent with the observation of Kagan and colleagues that oxidation of PS is necessary for its externalization (16, 22, 30); more recently, Tyurina et al. showed that in HL-60 cells exposed to nitrosative stress PS exposure was dissociated from the common apoptotic pathway (29). We have also recently reported that 24-h ascorbate-deficient neutrophils fail to undergo PS exposure and uptake by macrophages despite activation of caspases (33).

It has been proposed that the accumulation of neutrophils associated with the pathology of CGD is in part due to impaired clearance of these cells (8, 14, 19). Our observations now indicate that these neutrophils also have active caspases. One possibility is that caspase activation in these neutrophils could enhance the structural dismantling of the cell, increasing the potential for release of neutrophil proteins and exacerbation of local tissue damage. Such a phenomenon is consistent with the increased gastric atrophy observed in CGD mice colonized with *Helicobacter pylori* (17).

Many studies have used morphological changes to the neutrophil nucleus as a key marker of apoptosis (2, 18, 34, 35). However, we found that nuclei of phagocytic neutrophils underwent morphological changes clearly different from those of nuclei of neutrophils undergoing spontaneous apoptosis. Zhang and colleagues thought that morphological features of

apoptosis, such as nuclear condensation, could become distorted by an overabundance of yeast and therefore used only two yeast particles per human neutrophil (35). Although the loss of the characteristic multilobed nuclei could result from space constraints within the neutrophil, the same morphology was observed in neutrophils incubated with *S. aureus* at lower ratios containing only one or two bacteria within the cytoplasm. Coxon et al. have also reported NADPH oxidase-dependent changes in nuclear morphology in neutrophils phagocytosing serum-opsonized target particles (6), and Fuchs and colleagues recently described morphology distinct from apoptosis and necrosis in stimulated neutrophils during neutrophil extracellular trap formation that also showed irregular swollen nuclei and required oxidants derived from the NADPH oxidase (10). Therefore, we do not believe that nuclear morphological changes can be used to assess apoptosis in phagocytic neutrophils.

In summary, our studies indicate that the roles of oxidants generated by the neutrophil NADPH oxidase upon phagocytosis extend beyond the exclusive task of bacterial killing. We show that these neutrophil oxidants also serve as important signaling molecules in the phagocytic neutrophil. NADPH oxidase-derived oxidants trigger cell surface changes that result in macrophage recognition and engulfment of phagocytic cells. This phagocytic neutrophil clearance is a caspase-independent process; indeed, the neutrophil-derived oxidants prevent caspase involvement.

#### ACKNOWLEDGMENTS

This work was made possible by the award of a Sir Charles Hercus Fellowship from the Health Research Council of New Zealand to M.H. and of a Ph.D. scholarship from the University of Otago to R.W.

#### REFERENCES

- Baran, J., K. Guzik, W. Hryniewicz, M. Ernst, H. D. Flad, and J. Pryjma. 1996. Apoptosis of monocytes and prolonged survival of granulocytes as a result of phagocytosis of bacteria. *Infect. Immun.* **64**:4242–4248.
- Borjesson, D. L., S. D. Kobayashi, A. R. Whitney, J. M. Voyich, C. M. Argue, and F. R. DeLeo. 2005. Insights into pathogen immune evasion mechanisms: *Anaplasma phagocytophilum* fails to induce an apoptosis differentiation program in human neutrophils. *J. Immunol.* **174**:6364–6372.
- Brach, M. A., S. deVos, H. J. Gruss, and F. Herrmann. 1992. Prolongation of survival of human polymorphonuclear neutrophils by granulocyte-macrophage colony-stimulating factor is caused by inhibition of programmed cell death. *Blood* **80**:2920–2924.
- Cheah, F. C., M. B. Hampton, B. A. Darlow, C. C. Winterbourn, and M. C. Vissers. 2005. Detection of apoptosis by caspase-3 activation in tracheal aspirate neutrophils from premature infants: relationship with NF-kappaB activation. *J. Leukoc. Biol.* **77**:432–437.
- Colotta, F., F. Re, N. Polentarutti, S. Sozzani, and A. Mantovani. 1992. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* **80**:2012–2020.
- Coxon, A., P. Rieu, F. J. Barkalow, S. Askari, A. H. Sharpe, U. H. von Andrian, M. A. Arnaout, and T. N. Mayadas. 1996. A novel role for the beta 2 integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. *Immunity* **5**:653–666.
- Engelich, G., M. White, and K. L. Hartshorn. 2001. Neutrophil survival is markedly reduced by incubation with influenza virus and *Streptococcus pneumoniae*: role of respiratory burst. *J. Leukoc. Biol.* **69**:50–56.
- Fadeel, B., A. Ahlin, J. I. Henter, S. Orrenius, and M. B. Hampton. 1998. Involvement of caspases in neutrophil apoptosis: regulation by reactive oxygen species. *Blood* **92**:4808–4818.
- Fadok, V. A., J. S. Savill, C. Haslett, D. L. Bratton, D. E. Doherty, P. A. Campbell, and P. M. Henson. 1992. Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. *J. Immunol.* **149**:4029–4035.
- Fuchs, T. A., U. Abed, C. Goosmann, R. Hurwitz, I. Schulze, V. Wahn, Y. Weinrauch, V. Brinkmann, and A. Zychlinsky. 2007. Novel cell death program leads to neutrophil extracellular traps. *J. Cell Biol.* **176**:231–241.
- Hampton, M. B., A. J. Kettle, and C. C. Winterbourn. 1998. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* **92**:3007–3017.
- Hampton, M. B., and S. Orrenius. 1997. Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis. *FEBS Lett.* **414**:552–556.
- Hampton, M. B., I. Stamenkovic, and C. C. Winterbourn. 2002. Interaction with substrate sensitises caspase-3 to inactivation by hydrogen peroxide. *FEBS Lett.* **517**:229–232.
- Hampton, M. B., M. C. Vissers, J. I. Keenan, and C. C. Winterbourn. 2002. Oxidant-mediated phosphatidylserine exposure and macrophage uptake of activated neutrophils: possible impairment in chronic granulomatous disease. *J. Leukoc. Biol.* **71**:775–781.
- Hampton, M. B., M. C. Vissers, and C. C. Winterbourn. 1994. A single assay for measuring the rates of phagocytosis and bacterial killing by neutrophils. *J. Leukoc. Biol.* **55**:147–152.
- Kagan, V. E., B. Gleiss, Y. Y. Tyurina, V. A. Tyurin, C. Elenstrom-Magnusson, S. X. Liu, F. B. Serinkan, A. Arroyo, J. Chandra, S. Orrenius, and B. Fadeel. 2002. A role for oxidative stress in apoptosis: oxidation and externalization of phosphatidylserine is required for macrophage clearance of cells undergoing Fas-mediated apoptosis. *J. Immunol.* **169**:487–499.
- Keenan, J. I., R. A. Peterson, and M. B. Hampton. 2005. NADPH oxidase involvement in the pathology of *Helicobacter pylori* infection. *Free Radic. Biol. Med.* **38**:1188–1196.
- Kobayashi, S. D., K. R. Braughton, A. R. Whitney, J. M. Voyich, T. G. Schwan, J. M. Musser, and F. R. DeLeo. 2003. Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils. *Proc. Natl. Acad. Sci. USA* **100**:10948–10953.
- Kobayashi, S. D., J. M. Voyich, K. R. Braughton, A. R. Whitney, W. M. Nauseef, H. L. Malech, and F. R. DeLeo. 2004. Gene expression profiling provides insight into the pathophysiology of chronic granulomatous disease. *J. Immunol.* **172**:636–643.
- Li, Y., and M. A. Trush. 1998. Diphenyleneiodonium, an NAD(P)H oxidase inhibitor, also potently inhibits mitochondrial reactive oxygen species production. *Biochem. Biophys. Res. Commun.* **253**:295–299.
- Lundqvist-Gustafsson, H., S. Norrman, J. Nilsson, and A. Wilsson. 2001. Involvement of p38-mitogen-activated protein kinase in *Staphylococcus aureus*-induced neutrophil apoptosis. *J. Leukoc. Biol.* **70**:642–648.
- Matsura, T., B. F. Serinkan, J. Jiang, and V. E. Kagan. 2002. Phosphatidylserine peroxidation/externalization during staurosporine-induced apoptosis in HL-60 cells. *FEBS Lett.* **524**:25–30.
- Nilsdotter-Augustinsson, A., A. Wilsson, J. Larsson, O. Stendahl, L. Ohman, and H. Lundqvist-Gustafsson. 2004. *Staphylococcus aureus*, but not *Staphylococcus epidermidis*, modulates the oxidative response and induces apoptosis in human neutrophils. *APMIS* **112**:109–118.
- Perskvist, N., M. Long, O. Stendahl, and L. Zheng. 2002. Mycobacterium tuberculosis promotes apoptosis in human neutrophils by activating caspase-3 and altering expression of Bax/Bcl-xL via an oxygen-dependent pathway. *J. Immunol.* **168**:6358–6365.
- Pullar, J. M., and M. B. Hampton. 2002. Diphenyleneiodonium triggers the efflux of glutathione from cultured cells. *J. Biol. Chem.* **277**:19402–19407.
- Savill, J., and C. Haslett. 1994. Fate of neutrophils, p. 295–314. *In* P. J. Hellewell and T. J. Williams (ed.), *Immunopharmacology of neutrophils*. Academic Press, London, United Kingdom.
- Savill, J. S., A. H. Wyllie, J. E. Henson, M. J. Walport, P. M. Henson, and C. Haslett. 1989. Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J. Clin. Invest.* **83**:865–875.
- Sim, S., T. S. Yong, S. J. Park, K. I. Im, Y. Kong, J. S. Ryu, D. Y. Min, and M. H. Shin. 2005. NADPH oxidase-derived reactive oxygen species-mediated activation of ERK1/2 is required for apoptosis of human neutrophils induced by *Entamoeba histolytica*. *J. Immunol.* **174**:4279–4288.
- Tyurina, Y. Y., L. V. Basova, N. V. Konduru, V. A. Tyurin, A. I. Potapovich, P. Cai, H. Bayir, D. Stoyanovsky, B. R. Pitt, A. A. Shvedova, B. Fadeel, and V. E. Kagan. 2007. Nitrosative stress inhibits the aminophospholipid translocase resulting in phosphatidylserine externalization and macrophage engulfment: implications for the resolution of inflammation. *J. Biol. Chem.* **282**:8498–8509.
- Tyurina, Y. Y., F. B. Serinkan, V. A. Tyurin, V. Kini, J. C. Yalowich, A. J. Schroit, B. Fadeel, and V. E. Kagan. 2004. Lipid antioxidant, etoposide, inhibits phosphatidylserine externalization and macrophage clearance of apoptotic cells by preventing phosphatidylserine oxidation. *J. Biol. Chem.* **279**:6056–6064.
- van Zandbergen, G., J. Gieffers, H. Kothe, J. Rupp, A. Bollinger, E. Aga, M. Klinger, H. Brade, K. Dalhoff, M. Maass, W. Solbach, and T. Laskay. 2004. Chlamydia pneumoniae multiply in neutrophil granulocytes and delay their spontaneous apoptosis. *J. Immunol.* **172**:1768–1776.
- Vissers, M. C., W. A. Day, and C. C. Winterbourn. 1985. Neutrophils adherent to a nonphagocytosable surface (glomerular basement membrane) produce oxidants only at the site of attachment. *Blood* **66**:161–166.
- Vissers, M. C., and R. P. Wilkie. 2007. Ascorbate deficiency results in im-

- paired neutrophil apoptosis and clearance and is associated with up-regulation of hypoxia-inducible factor 1 $\alpha$ . *J. Leukoc. Biol.* **81**:1236–1244.
34. **Watson, R. W., H. P. Redmond, J. H. Wang, C. Condrón, and D. Bouchier-Hayes.** 1996. Neutrophils undergo apoptosis following ingestion of *Escherichia coli*. *J. Immunol.* **156**:3986–3992.
35. **Zhang, B., J. Hirahashi, X. Cullere, and T. N. Mayadas.** 2003. Elucidation of molecular events leading to neutrophil apoptosis following phagocytosis: cross-talk between caspase 8, reactive oxygen species, and MAPK/ERK activation. *J. Biol. Chem.* **278**:28443–28454.
36. **Zheng, L., M. He, M. Long, R. Blomgran, and O. Stendahl.** 2004. Pathogen-induced apoptotic neutrophils express heat shock proteins and elicit activation of human macrophages. *J. Immunol.* **173**:6319–6326.

---

*Editor:* F. C. Fang